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; Bacus et al. Molecular Carcinogenesis 3:350-362 (1990);

Stancovski et al. PNAS (USA) 88:8691-8695 (1991);

Bacus et al. Cancer Research 52:2580-2589 (1992)

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Kasprzyk et al. Cancer Research 52:2771-2776 (1992);

Hancock et al Cancer Res. 51:4575-4580 (1991);

Shawver et al. Cancer Res. 54:1367-1373 (1994);

Arteaga et al. Cancer Res. 54:3758-3765 (1994);

Harwerth et al. J. Biol. Chem.267:15160-15167 (1992);

Klapper et al. Oncogene 14:2099-2109 (1997)

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Semin Cancer Biol 7:79-86, 1996

Cancer res 52:2771-6, 1992

thank you,

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Requirements f r the Internalization of a Murine Monoclonal Antibody Directed against the HER-2/*neu* Gene Product c-*erb*B-2[1]

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ABSTRACT

A murine monoclonal antibody, TA1, is directed against an epitope on the extracellular domain of the HER-2/*neu* (c-*erb*B-2) gene product. Requirements for TA1-induced internalization of c-*erb*B-2 have been studied using the SKBr3 human breast cancer cell line and several rat fibroblast cell lines that express either wild-type or mutant human c-*erb*B-2. Internalization of TA1 was monitored by assaying protease-resistant uptake of ^{125}I-labeled TA1, by electron microscopy of gold-labeled TA1, and by inhibition of clonogenic growth of cells incubated with TA1 that had been conjugated with blocked ricin. Similar rates of internalization of TA1 were observed in SKBr3 and in rat fibroblasts that expressed human c-*erb*B-2. The route of endocytosis was the same as that observed with antibodies against other membrane receptors. Anti-c-*erb*B-2 and anti-transferrin receptor cointernalized through clathrin-coated pits, coated vesicles, endosomes, and multivesicular bodies. Products of mutant c-*erb*B-2 that lacked a portion of the tyrosine kinase domain or that lacked most of the cytoplasmic domain were endocytosed in the presence of TA1 as promptly as the wild-type c-*erb*B-2 product. Slightly more rapid internalization of TA1 was observed in rat cells that expressed c-*erb*B-2 with a single point mutation in the transmembrane domain. Taken together, our data suggest that neither the intracytoplasmic domain nor receptor phosphorylation is required for antibody-mediated endocytosis of c-*erb*B-2.

INTRODUCTION

The c-*erb*B-2 gene encodes a cell surface glycoprotein which is homologous to the EGFR.[3] This protein is composed of extracellular, transmembrane, and intracellular domains. The extracellular domain contains two cysteine-rich areas and is 44% homologous to EGFR (1). The intracellular domain contains a tyrosine kinase which is 82% homologous to that of EGFR. Because of these similarities to the EGFR and to other tyrosine kinase receptors, investigators have suggested that the c-*erb*B-2 protein may function as a growth factor receptor. A putative ligand for this receptor-like protein has recently been reported (2).

The oncogenic activity of the rat *neu* gene was initially associated with a point mutation from valine to glutamic acid in the transmembrane domain of neuroblastomas (3). In human cancers this same mutation has not been found in the c-*erb*B-2 gene, although substitution of the glutamic acid for valine at the same position in the transmembrane domain of the human gene followed by transfer into murine cells results in their transformation.[4] In clinical specimens, the wild-type c-*erb*B-2 gene is amplified and overexpressed in adenocarcinomas from several different sites, including breast, pancreatic, and ovarian cancers (4–7). A number of studies have reported an association between poor prognosis and overexpression of the protein in breast cancer (8, 9) and ovarian cancer (10, 11). Because of its overexpression in these carcinomas, the c-*erb*B-2 protein is a potentially useful target for therapy with monoclonal antibody conjugates that react with its extracellular domain.

Immunotoxins have been prepared that contain monoclonal antibodies conjugated to plant or bacterial toxins. Immunotoxins that contain ricin A chain or blocked ricin appear to be internalized through clathrin-coated pits and vesicles. The toxin moieties must then be released from vesicles into the cytoplasm to inhibit protein synthesis by catalytic inactivation of the 60S ribosomal subunit. The EGFR is an effective target for ricin A chain immunotoxins (12). During ligand-dependent and antibody-dependent internalization, EGFR can be found in clathrin-coated pits, vesicles, and endosomes (13, 14). Given the homology between EGFR and c-*erb*B-2, it seemed likely that the latter would be an effective target for an immunotoxin and that the conjugate would be internalized via the same pathway. To date, the internalization and subsequent fate of c-*erb*B-2 have not been fully studied, largely due to the absence of a well-defined ligand. In the case of EGF-mediated endocytosis of EGFR, the tyrosine kinase activity of the receptor may have some role, as receptors deficient in this activity may (14, 15) or may not (16, 17) be internalized efficiently. The intracellular domain of EGFR (18), as in the case of transferrin receptor (19–21), is important in facilitating the ligand-induced internalization of these cell surface molecules.

In this paper the antibody-dependent internalization of the c-*erb*B-2 protein, p185, was examined in a human breast cancer cell line that overexpressed c-*erb*B-2 as well as in different rat cell lines that expressed either wild-type or mutated human c-*erb*B-2 genes. These studies have helped to define the roles f the intracellular and transmembrane domains of c-*erb*B-2 in antibody-mediated endocytosis.

MATERIALS AND METHODS

Cell Lines. A human breast cancer cell line, SKBr3 (22), was maintained in RPMI 1640 medium supplemented with 15% FBS and 2 mM L-glutamine. The rat 1A, 1174, and 711 cell lines were obtained from Applied Biotechnology (Cambridge, MA). Rat 1A is a rat fibroblast cell line. The cell line 1174 was derived from rat 1A cells that had been infected with a defective retrovirus that contained the pMX1112 plasmid with the full-length wild-type human c-*erb*B-2. Immunoprecipitation (23) of 1174 cells with anti-c-*erb*B-2 antibodies yields the M_r 185,000 wild-type protein. Rat 711 cells had been similarly derived by infection with a defective retrovirus that contained the pMX1112 plasmid with the full-length human c-*erb*B-2 gene which had been mutated at codon 659 within the transmembrane domain substituting

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; FBS, fetal bovine serum; DMEM, Dulbecco's minimal essential medium; IgG, immunoglobulin G; IgM, immunoglobulin M; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DSP, dithiobis(succinimidylproprionate); EM, electron microscopy.

[4] A. Bruskin, unpublished data.

glutamic acid for valine (see Fig. 1). Immunoprecipitation of 711 cells with anti-c-*erb*B-2 antibodies yields a mutant M_r 185,000 protein. Rat 9-24c and 10-24k cells were derived from rat 1A cells by calcium-phosphate transfection with PMx1112 vector containing either a deletion of a portion of the tyrosine kinase domain (plasmid pABT9309) or deletion of all but 6 amino acids of the entire cytoplasmic domain (plasmid pABT9310), respectively. Immunoprecipitation of cells containing the pABT9309 construct yields a M_r 150,000 mutant c-*erb*B-2 protein.[5] Cells containing the pABT9310 construct express a M_r 100,000 mutant c-*erb*B-2 protein. The rat cell lines were maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine. In addition, medium for the 1174, 711, 9-24c, and 10-24k cells contained 400 μg/ml of G418 sulfate. All tissue culture reagents were obtained from GIBCO Laboratories, Grand Island, NY. The cells were cultured at 37°C in 5% CO_2 and 95% humidified air. For experiments, cells were detached with 0.25% trypsin-0.02% EDTA.

Antibodies and Immunotoxins. Hybridomas that produced the murine monoclonal antibodies TA1 (IgG1) and OD3 (IgM), known to react with the M_r 185,000 human c-*erb*B-2 product (23), but not with the rat *neu*, were obtained from Applied Biotechnology (Cambridge, MA). Three other murine monoclonal antibodies, MOPC21 (IgG1), 454A12 (IgG1), and 9C6 (IgM), were obtained from Cetus Corporation (Emeryville, CA). The 454A12 antibody reacts with the human transferrin receptor. MOPC21 and 9C6 antibodies failed to react with the rat cell lines or SKBr3 and were used as negative isotype-matched controls. An immunotoxin that contained the murine monoclonal antibody TA1 conjugated to ricin with a chemically altered B-chain that blocked binding to galactose (TA1-Br) was prepared by Immunogen (Cambridge, MA) and obtained through Applied Biotechnology. Unconjugated ricin with a blocked B-chain (Br) was also used as a control.

Plasmids. Plasmids pABT9309 and pABT9310 were obtained from Applied Biotechnology. pABT9309 was constructed from the pMX1112 plasmid and contained a human c-*erb*B-2 gene with a deletion of approximately 700 base pairs of the intracellular domain that included most of the coding sequence for the tyrosine kinase region (Fig. 1). pABT9310 was also constructed from pMX1112 and contained the coding sequence for all of the extracellular and transmembrane domain of the human c-*erb*B-2 gene, but lacked all but 6 amino acids of the intracellular domain. Plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (24).

Calcium Phosphate Transfection. Rat 1A cells were harvested and aliquots of 10^6 cells were plated in 100-mm Petri dishes. Cell cultures were incubated at 37°C overnight. The purified pABT9309 or pABT9310 plasmid DNA was diluted in 0.25 M calcium chloride. Nitrogen was bubbled into 2 ml of a solution of 19.6 ml of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with 280 mM sodium chloride and 0.425 ml of 70 mM dibasic sodium phosphate, pH 7.1. DNA was added dropwise to this mixture and incubated at room temperature for 40 min. A 2-ml portion of the DNA mixture was added to the rat 1A cultures (10 μg of DNA per 100-mm plate) and incubated at 37°C for 6 h. The medium was replaced, and the cultures were incubated for another 48 h. The cultures were split 1:4 in medium that contained 800 μg/ml of G418 sulfate. Individual colonies were selected in medium containing 800 μg/ml of G418 sulfate and subsequently maintained in medium containing 400 μg/ml of G418 sulfate.

Selection of Transfected Cells with Indirect Immunofluorescence and Flow Cytometry. Binding of murine monoclonal antibodies was measured by flow cytometry using a FITC-conjugated goat anti-mouse IgG (25). Transfected cells (5×10^5) were washed in PBS with 1% FBS and 0.02% sodium azide, incubated with dilutions of different antibodies for 30 min on ice, and then washed 3 times with assay buffer. The fluorescein-conjugated goat anti-mouse immunoglobulin was added to the cells for 30 min on ice. The cells were washed 3 times in assay buffer, resuspended in PBS, and analyzed with an Epics 753 flow cytometer (Coulter Electronics, Hialeah, FL). Clones transfected with pABT9309 or pABT9310 were chosen that exhibited binding of TA1 antibody comparable in fluorescence intensity to that of 1174 and 711 cells. The 9-24c clone contained pABT9309 that lacked a portion of the tyrosine kinase region, whereas the 10-24k clone contained pABT9310 that lacked all but 6 amino acids of the intracellular domain.

Radiolabeling of Antibodies. Antibodies were conjugated with $Na^{125}I$ obtained from Amersham (Arlington Heights, IL), using the Iodogen method as previously described (26).

Scatchard Analysis. The number of receptors per cell was estimated using different concentrations of immunoglobulins including those sufficient to saturate all binding sites (27). For the assay, 10^5 rat cells or 4×10^5 SKBr3 cells were seeded in 48-well plates and incubated overnight until cells were confluent. To block nonspecific binding of antibody, cells were incubated with 10% BSA in either DMEM or RPMI 1640 for 1.5 h at 37°C. ^{125}I-labeled antibody was added to triplicate aliquots of cells at final concentrations of 2.5, 0.5, 0.25, 0.05, and 0.005 μg/ml. After 2-h incubation at 37°C, cells were washed 3 times in DMEM or RPMI 1640, lysed with 2 N NaOH, and counted in a Packard gamma counter. Aliquots of ^{125}I-labeled antibody were also counted. The number of cells in each microtiter well was estimated by detaching monolayers from 6 wells using trypsin and counting the cells in the presence of 0.1% trypan blue using a hemocytometer.

Internalization of ^{125}I-TA1. ^{125}I-labeled TA1 (1 μg/ml) was added to aliquots of 5×10^5 rat cells. After incubation for 1 h at 4°C to allow binding, cells were washed 3 times with DMEM with 1% BSA and then either counted immediately to determine the total amount of antibody bound or incubated at either 4°C or 37°C for 1 to 4 h to permit internalization of immunoglobulin. To remove antibody still bound to the cell surface, 2.5 mg/ml of proteinase K (Sigma) was added to the cells for 1 h at 37°C. The cells were washed 3 times in DMEM with 1% BSA and 0.1% sodium azide. Radioactivity associated with cell pellets was counted in a Packard gamma counter. The amount (cpm) of antibody internalized was determined by subtracting the cpm obtained after incubation at 4°C followed by protease stripping from the cpm obtained after incubation at 37°C for the same time interval, followed by protease stripping. The percentage internalized was then calculated by dividing the cpm of the antibody internalized by the total cpm initially bound to the cell surface. Protease stripping immediately after binding removed an average of 94 to 98% of the surface-bound immunoglobulin from the various transfectant cell lines in three experiments.

Assays of Clonogenic Growth. To study the effect of TA1-bR on growth of cells that expressed c-*erb*B-2, a clonogenic assay was used. Rat fibroblasts (5×10^5) and SKBr3 cells (1×10^6) were incubated at 37°C on a rotator with different concentrations of the TA1-bR immunotoxin conjugate or unconjugated bR as a control for 6, 12, 18, or 24 h. The cells were washed twice with tissue culture medium and serially diluted 5-fold. A 100-μl portion of each dilution was plated in each of 6 wells within a 96-well flat-bottomed microtiter plate. An additional 100-μl tissue culture medium was added to each well. The cells were incubated for 7 days at 37°C. Clonogenic growth was determined using an inverted phase microscope, scoring the number of wells with at least one tumor colony that contained at least 10 cells. Estimates of the surviving clonogenic units were calculated according to a modification by Johnson and Brown (28) of the method of Spearman and Karber. Diluent-treated cultures were used as controls for the assay. Clonogenic elimination was calculated by subtracting the log clonogenic units of the diluent-treated cultures from the log clonogenic units of the treated cell culture for each individual cell line.

Conjugation of Antibody with Gold Sols. Five- and 15-nm gold sols were obtained from Amersham (Arlington Heights, IL) for conjugation to monoclonal antibodies according to instructions of the manufacturer. Briefly, the antibodies were dialyzed against 2 mM sodium borate buffer, pH 9.0, and centrifuged at $100,000 \times g$ for 1 h. The upper two-thirds of the supernatant was retained. The antibody concentration was estimated using A_{280}. The pH of the gold sols was adjusted to approximately 0.5 units above the pI of the antibody. To prepare the gold probe, 25 ml of pH-adjusted beads were added to 17 to 40 μg/ml of antibody and stirred for 2 min. The pH was adjusted to 9.0 with potassium carbonate. A final concentration of 1% BSA was added, using a solution of 10% BSA in distilled deionized water. To remove any unconjugated gold sol, the mixture was centrifuged at 4°C for 45 min at $45,000 \times g$ for

[5] S. McKenzie, unpublished data.

the 5-nm sol or at 12,000 × *g* for the 15-nm sol. The pellet was resuspended in the lower 10% of the supernatant and layered over a 10 to 30% glycerol step gradient. The gradient was centrifuged for 45 min at 125,000 × *g* for the 5-nm sol or at 15,000 × *g* for the 15-nm sol. The concentrated conjugated gold probes were then dialyzed against 1% BSA-Tris buffer, pH 8.2, to remove the glycerol, and the absorbance was measured at 520 nm.

Immunoelectron Microscopy. Confluent SKBr3 cells in 60-mm Petri plates were incubated with 0.4 to 0.6 ml of gold-conjugated TA1 and 454A12 alone or in combination for 30 min at 4°C. Concentrations of conjugates were estimated by A_{520} that ranged from 1.7 to 3.0 for 15-nm conjugates and from 0.8 to 1.4 for 5-nm conjugates. Confluent rat 1A, 1174, 711, 9-24c, and 10-24k cells were incubated with 0.4 ml of TA1-gold conjugate for 30 min at 4°C. The cells were then washed with PBS to remove any nonadherent antibody and were warmed to 37°C for 0, 10, or 30 min to allow internalization. The cells were fixed for at least 1 h with 2% glutaraldehyde in 150 mM sodium cacodylate buffer at pH 7.4 with 2.5 mM calcium chloride. The fixed cells were scored with a blade and removed from the dishes with a rubber policeman. Monolayers were sedimented by centrifugation. The pellets were postfixed on ice with 2% osmium tetroxide and 0.5 to 1.0% potassium ferrocyanide in the same buffer. After washing in cacodylate buffer, cells were transferred to sodium acetate buffer, and the pellets were stained *en bloc* with 1% uranyl acetate in 0.2 M sodium acetate, pH 5.2. Subsequently, cells were dehydrated with increasing concentrations of ethanol, incubated with mixtures of 100% ethanol and epoxy, and embedded in beem capsules with Embed 812 (EM Sciences).

Pale gold to silver sections were cut on a Reichert-Jung Ultracut E microtome and stained with either saturated uranyl acetate and lead citrate or with lead citrate alone. The sections were examined on a Phillips 300 electron microscope at 80 kV.

Cross-Linking and Immunoprecipitation of Cell Surface Molecules. 10-24k cells were radiolabeled overnight with [^{35}S]cysteine (Amersham) and treated with either diluent or the reducible cross-linking agent DSP (Pierce). Cell lysates were immunoprecipitated (23) with either an irrelevant IgG (MOPC-21) or an anti-c-*erb*B-2 monoclonal antibody (TA1) and Protein A-Sepharose. Immunoprecipitates were eluted with sodium citrate (pH 2.8), analyzed under reducing and nonreducing conditions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiographed.

RESULTS

Expression of Wild-Type and Mutant c-*erb*B-2 in SKBr3 and in Rat 1A Fibroblasts. Requirements for antibody-mediated internalization of c-*erb*B-2 were studied with cell lines that expressed the wild-type (SKBr3, 1174) or modified gene products (711, 9-24c, and 10-24k) (Fig. 1). Rat 1 fibroblasts lacked expression of the c-*erb*B-2 product and had served as the parent line for transfection of constructs. The 9-24c line expressed c-*erb*B-2 that lacked the tyrosine kinase coding region, whereas the 10-24k expressed c-*erb*B-2 from which all but 6 amino acids of the intracellular domain had been deleted. The 711 line contained a construct in which a point mutation had been inserted at codon 659 within the transmembrane domain.

Expression f c-*erb*B-2 was compared using indirect immunofluorescence after binding of the TA1 murine monoclonal antibody to the extracellular domain of the gene product. As indicated in Fig. 2, the 10-24k cells displayed the highest level of fluorescence. The 711 cells, while displaying a significant level of fluorescence, were the least reactive with antibody. The 1174 and 9-24c cells displayed levels of fluorescence intermediate in intensity between the 10-24k and 711 cells.

The c-*erb*B-2 expression of each cell line was confirmed by immunoprecipitating the protein with TA1 (data not shown). The SKBr3, 1174, and 711 cells expressed M_r 185,000 products when precipitated with TA1. The 9-24c line expressed a M_r 150,000 product and 10-24k, a M_r 100,000 product, consistent with predictions from the constructs.

The number of c-*erb*B-2 molecules present per cell was estimated by Scatchard analysis for each of the rat fibroblast cell lines and the SKBr3 cell line. As indicated in Table 1, the K_a for TA1 was similar for the rat fibroblast cell lines. The SKBr3 cells had the highest concentration of sites, with 1.3×10^6 molecules per cell. Similar levels of c-*erb*B-2 have been reported for SKBr3 using different antibodies against c-*erb*B-2 to estimate antigen binding sites (29). The rat fibroblasts displayed a lower level of c-*erb*B-2 expression than did the SKBr3 cells, ranging from 2.3×10^4 sites/cell for the 1174 cells to 9.5×10^4 sites/cell for the 10-24k cells. This analysis indicates that all 4 transfected cell lines expressed similar concentrations of c-*erb*B-2 molecules per cell, with slightly higher expression by the 10-24k cells.

Endocytosis of ^{125}I-labeled TA1. To determine if the 4 rat cell lines internalized TA1 at the same rate, ^{125}I-labeled antibody was added to the cells for 1 h at 4°C to permit binding. The cells were subsequently warmed to 37°C for different intervals to allow internalization. Antibody that had not been internalized was removed with proteinase K. In preliminary experiments, minimal internalization of ^{125}I-labeled antibody was observed at 15- and 30-min intervals at 37°C. Therefore 1-h and 4-h intervals were tested. ^{125}I-TA1 activity that resisted proteinase K digestion in 3 replicate experiments is plotted in Fig. 3. Rat 1A cells do not express the human c-*erb*B-2 and did not internalize the TA1 antibody. ^{125}I-TA1 bound to each of the rat transfectants, but only 6 to 12% of the antibody that bound to cells was internalized after 4 h. There was no significant difference between the 4 rat cell lines in the percentage of the antibody internalized at 1 or 4 h (*t* test, $P > 0.05$). Among the 4 cell lines, uptake by 711 was somewhat more prompt than uptake by the other lines. After 1 h at 37°C, 12% of the antibody that had bound to the 711 cells was internalized, whereas only

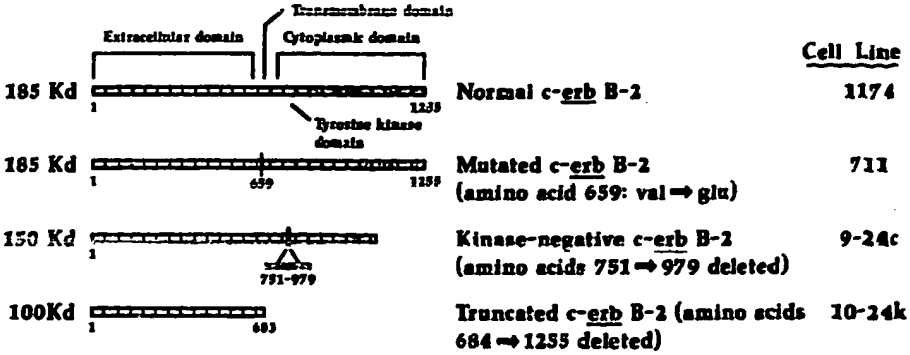

Fig. 1. The structures of the c-*erb*B-2 gene constructs in the pmx1112 expression vector from *top* to *bottom* include: the full-length wild-type c-*erb*B-2 gene retrovirally transferred into 1174 cells; full-length c-*erb*B-2 with one amino acid mutation at codon 659 retrovirally transferred into the rat 1A cells to yield 711 cells; kinase-negative c-*erb*B-2 with a deletion of amino acids 751 to 979 transfected into the rat 1A cell to yield 9-24c cells; and a c-*erb*B-2 construct lacking most of the intracytoplasmic domain, leaving a cytoplasmic tail of 6 amino acids transfected into the rat 1A cell, to yield 10-24k cells.

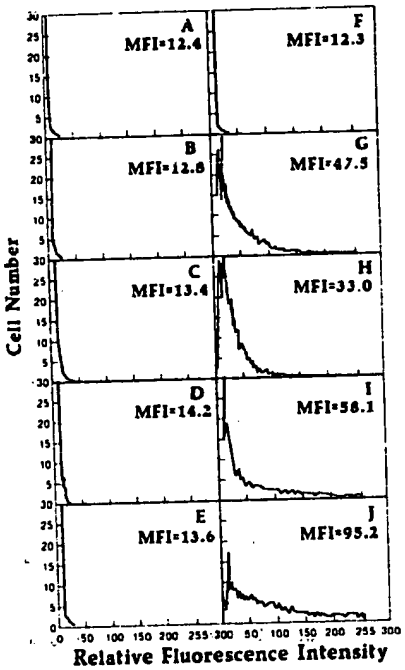

Fig. 2. Flow cytometric analysis of TA1 binding (5 μg/ml) to the rat cell lines, using a FITC labeled goat anti-mouse antibody and isotype matched negative control, MOPC21 (5 μg/ml). Rat 1A cells with MOPC21 (*A; 1.2% cells positive*) or TA1 (*F; 0.9% cells positive*). 1174 cells with MOPC21 (*B; 1.4% cells positive*) or TA1 (*G; 81.5% cells positive*). 711 cells with MOPC21 (*C; 17.2% cells positive*) or TA1 (*H; 82.8% cells positive*). 9-24c cells with MOPC21 (*D; 3.2% cells positive*) or TA1 (*I; 68.9% cells positive*). and 10-24k cells with MOPC21 (*E; 1.7% cells positive*) or TA1 (*J; 93.0% cells positive*). MFI (Mean Fluorescence Intensity) is expressed as channel number on a linear scale.

Table 1 *c-erbB-2 sites per cell and association constants determined by Scatchard analysis with ^{125}I-TA1*

Cell line	Sites/cell	K_a (M^{-1})
1174	2.3×10^4	11.2×10^8
711	5.3×10^4	5.1×10^8
9-24c	4.2×10^4	8.2×10^8
10-24k	9.5×10^4	3.8×10^8
SKBr3	1.3×10^6	8.7×10^7

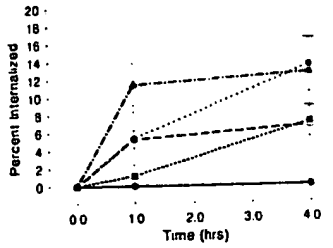

Fig. 3. Internalization of ^{125}I-labeled TA1 by different rat cell lines: rat 1A (——); 1174 (– – –); 711 (– · –); 9-24 C (· · · · ·); and 10-24k (– – – –). *Points*, mean percentage internalized of three experiments; *bars*, SE.

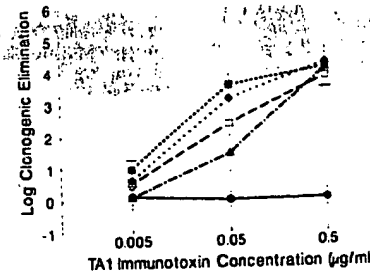

Fig. 4. Clonogenic elimination of rat transfectants with TA1-bR immunotoxin following a 24-h incubation. ——, rat 1A; – – –, 1174; – · –, 711; · · · · ·, 9-24c; – – – –, 10-24k. *Points*, mean log clonogenic elimination of three experiments; *bars*, SE.

1 to 2% of bound antibody was internalized by 1174 cells. This difference at 1 h approached statistical significance ($P = 0.065$), but by 4 h there was no apparent difference in the amount of labeled TA1 internalized by the two cell lines. The 9-24c and 10-24k cell lines showed a level of internalization intermediate between 1174 and 711 cells at 1 h, and uptake by each cell line was approximately the same by 4 h (Fig. 3).

Endocytosis of TA1-blocked Ricin Immunotoxin Conjugates. To assess further the internalization of TA1, each of the rat cell lines was treated with TA1 immunotoxin. Only the antibody conjugate that was internalized would inhibit clonogenic growth in a limiting dilution assay. Unconjugated blocked ricin was used on an equimolar basis as a control for the conjugate and did not affect growth of any of the cell lines at the concentrations of toxin used in this study (data not shown). As indicated in Fig. 4, a 24-h incubation with the TA1 immunotoxin inhibited the growth of all 4 rat transfectant cell lines, but did not affect growth of the rat 1A cell line that lacked c-*erb*B-2.

Cells with more complete or more rapid internalization of the immunotoxin should display greater inhibition of clonogenic growth than cells with less complete or less rapid internalization of immunotoxin. To study the kinetics of internalization and elimination of clonogenic cells by TA1 immunotoxin, the 1174 and 10-24k cell lines were treated with the TA1-bR conjugate for different intervals from 6 to 24 h. With each of the cell lines, the longer the incubation with immunotoxin, the greater the fraction of cells that were killed (Fig. 5). Each of the cell lines including 9-24c and 711 (data not shown) exhibited similar sensitivity to the immunotoxin, consistent with similar rates and levels of internalization of TA1-bR after binding to c-*erb*B-2.

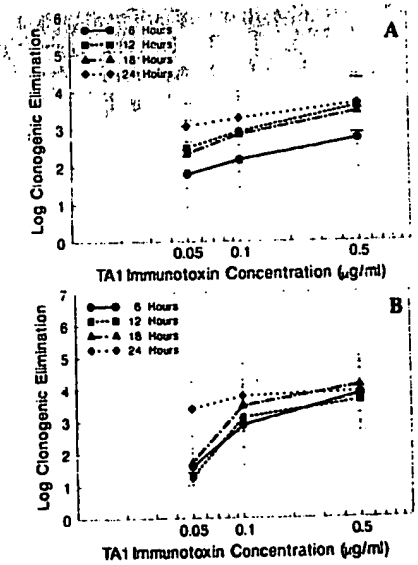

Fig. 5. *A*, kinetics of elimination of clonogenic 1174 cells with TA1 immunotoxin. *Points*, mean of log clonogenic elimination for three experiments; *bars*, SE. *B*, kinetics of elimination of clonogenic 10-24k cells with TA1 immunotoxin. *Points*, mean of log clonogenic elimination for 3 experiments; *bars*, SE.

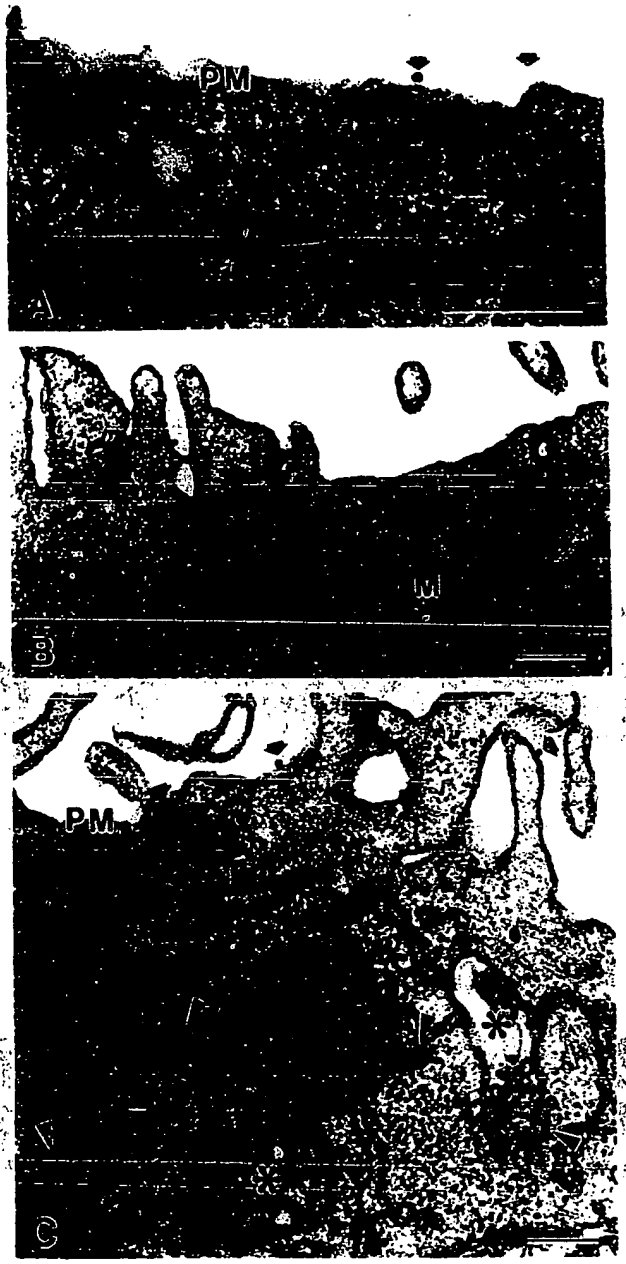

Fig. 6. Endocytosis of wild-type c-*erb*B-2 protein (p185) and transferrin receptor in SKBr3 breast carcinoma cells. SKBr3 cells were incubated with TA1-gold (15 nm) and 454A12-gold (5 nm), at 4°C, to label surface p185 and transferrin receptor, respectively. Endocytosis of the two receptors was followed at 37°C after 5 min (*A* and *B*) or 30 min (*C*). Clathrin-coated pits and endosomal vesicles contain both labels (*curled arrows* in *A* and *B*). Note the concentration of p185 label around surface microvilli (*B*). *C*, after 30 min of endocytosis most surface label is due to p185 (*broad arrows*), and multivesicular bodies are doubly labeled (*). The c-*erb*B-2 product is also present in small, tubular endosomes (*arrowheads*). *PM*, plasma membrane; *M*, mitochondria. *Bars*, 0.25 μm.

Endocytosis of Gold-TA1 Conjugates. To study the route by which the c-*erb*B-2 gene product (p185) is internalized, TA1 was conjugated to colloidal g ld particles. The gold-TA1 conjugate was added to cells at 4°C to allow binding, after which the cells were warmed t 37°C for 5, 10, and 30 min to allow endocytosis bef re fixation and secti ning for EM. After incubation at 4°C, the TA1 gold bound diffusely over the apical surface of each SKBr3 cell, consistent with the pattern seen by immun fluorescence. After warming for 5 min, some TA1-gold was observed in clathrin-c ated pits, vesicles, and superficial endosomes, although a significant portion of the tracer remained on the cell surface (Fig. 6, *A* and *B*). Anti-transferrin recept r antibody (454A12) c njugated to 5-nm gold particles was used as a marker f r clathrin-coated pits and vesicles, as transferrin is known to be internalized through these structures. TA1-gold and 454A12-gold conjugates coexist in pits and vesicles (Fig. 6*A*), further supporting the conclusion that the TA1 and p185 were internalized through clathrin-coated structures. By 30 min at 37°C, the TA1-gold conjugates were seen in deeper cellular structures, including endosomes, lysosomes, and multivesicular bodies (Fig. 6*C*). Much TA1-gold was still evident on the cell surface even after 30 min of endocytosis, a time when very little 454A12-gold remained at the cell surface (Fig. 6*C*). Thus, c-*erb*B-2 was internalized at a slower rate and to a lesser extent than the transferrin receptor.

The antibody-induced internalization of p185 in rat-1 fibroblasts resembled that in SKBr3 breast carcinoma cells. As expected, 1174 cells that expressed the wild-type human c-*erb*B-2 bound much less TA1 gold than did SKBr3 cells, consistent with the Scatchard analysis, whereas no binding was observed to rat-1 cells that had not been transfected. After 5 to 10 min at 37°C, TA1-gold was seen primarily on the surface of the 1174 fibroblasts. Occasionally, TA1-gold was present in clathrin-coated pits, vesicles, or superficial endosomes (Fig. 7). By contrast to the kinetics of 1174 endocytosis, after 5 min at 37°C much of the TA1-gold conjugate was found within 711 cells, primarily in peripheral clathrin-coated pits, vesicles, and endosomes, but also in some deep endosomes (Fig. 8, *A* to *C*). By 30 min at 37°C, most of the TA1-gold was internalized by 711 cells and was found in endosomes and in multivesicular bodies (Fig. 8*C*). After 30 min at 37°C, the TA1-gold in the other 3 cell lines was still primarily on the cell surface and in endosomal structures (Fig. 7*D*). Although the tracer was present in some endosomes adjacent to the Golgi complex in all of the rat cell lines, no gold was evident in the Golgi cisternae of any of the cells.

To determine whether the intracellular domain of the c-*erb*B-2 was necessary for internalization of the protein, we studied the 10-24k and 9-24c cell lines. As shown in Fig. 8, *D* and *E*, the truncated c-*erb*B-2 products were internalized in both cell lines, through the same route, and apparently with the same kinetics as the wild-type c-*erb*B-2 expressed in 1174 cells.

This observation was surprising, considering the role that the intracellular domain of EGFR may play in the endocytosis of that receptor (16, 17). Since p185 can, under some circumstances, associate with EGFR, we investigated the possibility that the truncated c-*erb*B-2 gene product was internalized as a complex with the rat EGFR. The 10-24k cells were labeled uniformly with [^{35}S]cysteine, and detergent lysates were immunoprecipitated first with TA1 and then with rabbit anti-rat EGFR (the generous gift of Dr. Sheldon Earp), to test for coprecipitation of proteins. We failed to detect M_r 185,000 or 170,000 bands in the TA1-eluted material, even after gross overexposure of the M_r 160,000 truncated c-*erb*B-2 band (p100). Association f p100 with other molecules was not observed, even when the labeled cells were subjected to DSP cross-linking (data not shown). It therefore seems unlikely that the truncated c-*erb*B-2 proteins were internalized thr ugh association with intact homologous receptors.

Fig. 7. Endocytosis of wild-type c-*erb*B-2 protein in fibroblasts. 1174 cells that express wild-type c-*erb*B-2 protein were incubated with TA1-gold (15-nm particles) at 4°C. They were then warmed to 37°C for different times, fixed, and processed for EM. *Arrowheads* highlight the monoclonal antibody-gold particles. *A* and *B*, cells after 5 min at 37°C, showing the label in clathrin-coated pits and vesicles. *C*, cells after 5 min at 37°C showing the random distribution of monoclonal antibody-conjugated gold particles over the plasma membrane. *D*, cells after 30 min of endocytosis. Note the label in endosomes (*) as well as on the cell surface. *M*, mitochondria; *PM*, plasma membrane. *Bars*, 0.5 μm.

DISCUSSION

In this paper, an antibody directed against the extracellular domain of the c-*erb*B-2 (HER-2/*neu*) gene product was used to examine the internalization of p185. Antibody-mediated endocytosis of p185 was studied in human breast carcinoma cells that expressed a large amount of the protein and in rat fibroblasts that expressed different mutants of the p185. Internalization was monitored by three assays that produced concordant results, including protease-resistant uptake of ^{125}I-TA1 antibody, inhibiti n of clonogenic growth by a TA1-toxin conjugate, and electron micr scopy of gold-labeled TA1.

Our data demonstrate that p185 is internalized by the pathway for end cytosis utilized by ther recept rs. In the presence f antibody, p185 passes thr ugh clathrin-coated pits and vesicles int a variety of endosomal structures. Later, the protein is found in deep endosomes and multivesicular bodies near the Golgi complex. Structurally, the multivesicular bodies seem equivalent to the prelysosomal compartment where many ther surface receptors, including the homologous EGFR, are f und.

TA1 antibody conjugated with c lloidal gold was used to map this pathway of internalizati n. The route of p185 endocytosis could be altered by the multivalent gold c njugate to direct more of the receptor into the prelysosomal compartment and to lysosomes, as has been shown for the endocytosis of gold-conjugated transferrin (30). This does not seem to be a problem in the present study, however, as significant routing f p185 into lysosomes was not detected over the time course f these experiments, in either SKBr3 cells or transfected fibroblasts.

The internalization of p185 appears to occur rather slowly in each of the cell lines, judged by protease-resistant uptake of ^{125}I-TA1 or by electron microscopy. In addition, the extent of endocytosis was modest. Other proteins are taken up more promptly and more completely than the c-*erb*B-2 gene product. Our EM studies show that the endocytosis of the transferrin receptor in SKBr3 cells is quite rapid, consistent with the half-time of 7 to 15 min for internalization of the transferrin receptor that had been measured in other cell types (31).

Many studies of receptor internalization have utilized specific ligands to trigger receptor translocation and uptake. This paper concerns only studies of antibody-mediated uptake performed in the absence of a readily available ligand for the c-*erb*B-2 gene product. Antibody-mediated internalization may not, of course, reflect the pattern of internalization when ligand binds to p185. Antibodies to EGFR have been described that do not cause internalization of the receptor (32), in direct contrast to the consequences of the EGF ligand binding to the EGFR. Antibodies can induce aggregation of cell surface receptors. Aggregation of p185 is thought to be important for activation (33). When judged by direct immunofluorescence, the TA1 antibody does not induce capping of p185, and only modest clustering of p185 has been observed on the cell surface.* Immunoelectron microscopy has also failed to show patching of p185. Thus, if aggregation is important for internalization, it must be on a scale not detectable by microscopy. Dimerization has also been shown to play a role in activation (34), but our morphological methods cannot distinguish monomeric from dimeric forms of the receptor.

Because the rat-1 cells express endogenous rat p185 as well as rat EGFR, it is possible that much of the exogenous human protein forms heterodimers with endogenous rat receptors. Internalization of human p185 observed in the presence of TA1 antibody might then reflect internalization of the endogenous rat proteins. If this were the case, endocytosis of the mutant p100 that lacked the intracellular domain could occur due to dimerization of the mutant gene product with normal rat receptors that maintained kinase activity and phosphorylation sites. Against this possibility, neither rat c-*erb*B-2 nor rat EGFR could be coprecipitated with human c-*erb*B-2 mutants expressed in the rat fibroblasts, even when a cross-linker was used to stabilize potential dimers. Consequently, endocytosis of the human p185 in all probability reflects properties of this protein alone.

Products of two c-*erb*B-2 mutants with deletions in the intracellular domain were internalized as rapidly as the wild-type c-*erb*B-2 gene product. Consequently, it seems that this receptor can be endocytosed in the absence of its cytoplasmic domain, in contrast to other transmembrane proteins whose intracellular

* M. Lien and Y. Argon, unpublished results.

Fig. 8. Endocytosis of mutant c-*erb*B-2 proteins. 711, 9-24c, and 10-24k cells were incubated with TA1-gold as described for Fig. 7. *A* to *C*, 711 cells expressing c-*erb*B-2 with a point mutation in the transmembrane domain, after endocytosis for 5 min (*A* and *B*) or 30 min (*C*). Note the presence of label in clathrin-coated pits, in peripheral endosomes, in deep endosomes, and in multivesicular bodies (*). A higher fraction of gold particles was consistently noted in these internal structures than on the plasma membrane (*PM*) after 5 min of endocytosis, as compared with the 1174 cell line (compare with Fig. 7). *D*, 10-24k cells after 30 min of endocytosis. *E*, 9-24c cells after 30 min of endocytosis. *Bars*, 0.5 μm.

domains are important for internalization through clathrin-coated pits and vesicles (15, 18–20, 35–37). Specific tyr sine residues, located 4 to 50 amin acids from the membrane spanning d main, appear t be required for clustering f transferrin receptor, low-density lipoprotein receptor, and other proteins in clathrin-coated pits (35). Mutati ns that delete or change cytoplasmic domains have produced aberrant traffic of membrane proteins and have prevented their internalization (16, 17). It is therefore surprising that antibody-dependent internalization of the c-*erb*B-2 gene product does not require the intracellular domain of p185. The substitution of glutamic acid for valine at codon 659 in the transmembrane domain of p185 does not inhibit and may facilitate antibody-mediated internalization. Uptake of the mutant p185 by 711 cells was enhanced, but this cell line was not more sensitive to immunotoxin. The antitumor activity of ricin, however, depends not only on the rate of internalization of the immunotoxin but also upon the rate of degradation of the immunotoxin and the rate of release of ricin into the cytoplasm. There is some evidence that substitution at codon 659 causes constitutive activation of the c-*erb*B-2 tyrosine kinase (33, 38). If phosphorylation of intracellular substrates enhances internalization, the 711 construct may be internalized more efficiently than the 10-24c kinase-negative mutant. Future studies will address the relationship between the kinetics of internalization of the c-*erb*B-2 gene product and phosphorylation of p185 as well as other intracellular substrates of the tyrosine kinases.

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